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Hydroxylation of Proline in vitro I

Hydroxyproline Formation in the System Containing Hydrogen Peroxide

By JOSEF HURYCH

*Department of Experimental Biology, Institute of Industrial Hygiene and Occupational Diseases, Prague**

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Summary: The conversion of proline into hydroxyproline in vitro was studied. The hydroxylation system consists of bivalent iron bound in a complex, ascorbic acid and hydrogen peroxide. In total, 4 isomers of hydroxyproline (4-*cis* and 4-*trans*, 3-*cis*- and 3-*trans*-) were isolated. It was found that hydroxylation takes place also in the absence of metal ions. Effective components in this case are probably free radicals formed by the reduction of hydrogen peroxide by ascorbic acid. Ascorbic acid increases the conversion of proline into hydroxyproline, but is not an essential part of the reaction system and can be replaced by an increased amount of iron. There is a non-specific hydroxylation caused by free radicals. The iron ions can be replaced by $Cu^{\oplus}$ or $Cr^{3\oplus}$. In the model mixture hydroxylation of peptide-bound proline also takes place. The amount of hydroxyproline formed depends on the position of the proline residue and on the length of the chain. Hydroxyproline is also formed in aqueous solution of proline which has been irradiated by a cobalt bomb.

Zusammenfassung: Die Umwandlung von Prolin in Hydroxyprolin wurde in vitro in einem Hydroxylierungssystem aus komplexgebundenem 2wertigem Eisen, Ascorbinsäure und Hydroperoxid untersucht. Insgesamt 4 Isomere (4-*cis*-, 4-*trans*-, 3-*cis*- und 3-*trans*-) wurden isoliert. Auch in Abwesenheit von Metallionen fand Hydroxylierung statt, wahrscheinlich durch Radikale, die bei der Reduktion von Hydroperoxid durch Ascorbinsäure entstehen. Ascorbinsäure erhöht die Umwandlungsrate, kann aber durch eine größere Eisen(II)-Konzentration ersetzt werden. Die gleiche Wirkung wie Eisen(II)-Ionen haben Kupfer(I)- und Chrom(III)-Ionen, also oxydierbare Metallionen. Im verwendeten System findet auch eine Hydroxylierung von peptidgebundenem Prolin statt. Die Menge gebildeten Hydroxyprolins hängt von der Stellung des Prolins im Peptid und von der Länge der Kette ab. Außerdem wird Hydroxyprolin in wäßriger Prolinlösung durch radioaktive Bestrahlung (Cobaltbombe) gebildet.

The unique appearance of hydroxyproline in collagenous proteins inspires the concept that the hydroxylation of the pyrrolidine ring will have a basic importance for the study of collagen biosynthesis. This hypothesis is further supported by the finding[1] that collagenous hydroxyproline originates from proline whereas exogenous applied hydroxyproline is not incorporated into collagen.

UDENFRIEND et al.[2,3] described in 1954 a system which is able to hydroxylate several aromatic and heterocyclic compounds. This system consists of bivalent iron complex bound by ethylenediaminetetraacetic acid, ascorbic acid and hydrogen peroxide. This medium was used for hydroxylation of kynurenine[4], steroids[5] and aliphatic hydrocarbons[6]. When studying amino acid reactions in an analogous system or after γ-irradiation it was found,

* Address: Dr. J. HURYCH, Šrobárova 48, Praha 10, Czechoslovakia.

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that decarboxylation and oxidative deamination[7,8] occurs particularly with formation of aldehydes, aldehydic-, carboxylic- or keto-carboxylic acids, further phenylalanine hydroxylation[9], amino acid conversion to a lower number of carbon atoms or opening of a cyclic ring[10]. It appears that the hydroxylation reactions are only a part of the more general oxidation reaction.

We have shown in our previous preliminary communications[11,12] that this system is able to hydroxylate the saturated pyrrolidine ring. Some of our results were confirmed later in other laboratories[13,14]. This paper deals with a detailed investigation of conditions for proline hydroxylation with the aim of using these findings in collagen biosynthesis.

Results

The rate of formation and decomposition of hydroxyproline was studied. The major amount of hydroxyproline is formed during the first 30 seconds (Fig. 1, curve 1). If the proline in the incubated mixture is replaced by hydroxyproline a decomposition of about 30% of the hydroxyproline present takes place (curve 2). Accordingly, the newly formed hydroxyproline is the result of synthetic and degradation processes which are caused, as is generally assumed[15,16], by free radicals:

$$Fe(II)[EDTA] + H_2O_2 \rightarrow Fe(III)[EDTA] + HO^{\ominus} + \cdot OH$$

Hydroxylation takes place in the absence of ascorbic acid to a negligible extent (curve 3). Further

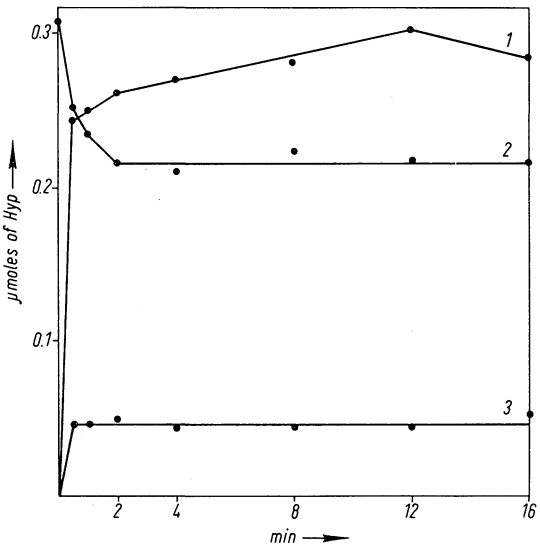

Fig. 1. Formation and decomposition of hydroxyproline with time. Curve 1: 20 µmoles proline incubated in the complete hydroxylation system; Curve 2: 0.305 µmoles hydroxyproline incubated in the complete hydroxylation system; Curve 3: 20 µmoles proline in the same system without ascorbic acid.

we solved the question of whether besides the normal 4-*trans*-hydroxyproline some other isomers are formed. For this reason we chromatographed the reaction mixture on Dowex 50WX8 and isolated all 4 isomers of hydroxyproline (Fig. 2). The data of the participation of the individual isomers are given in Table 1. The position of these substances in the effluent was identified by MOORE and STEIN's ninhydrin-method[17] (measured at 420 mµ) and STEGEMANN's method[18]. From the total amount of proline present in the medium approximately 25% react; of this about 30% serve as substrate for hydroxylation. The loss is due to the further degradation similarly as in other amino acids[8,10].

It was found that the conversion of proline into hydroxyproline was uninfluenced by temperature within the range of 25°—45°. The influence of the buffer composition on the extent of conversion was also investigated. The maximum hydroxylation of proline occurs in the phosphate buffer and the least suitable seem to be acetate and phthalate buffers

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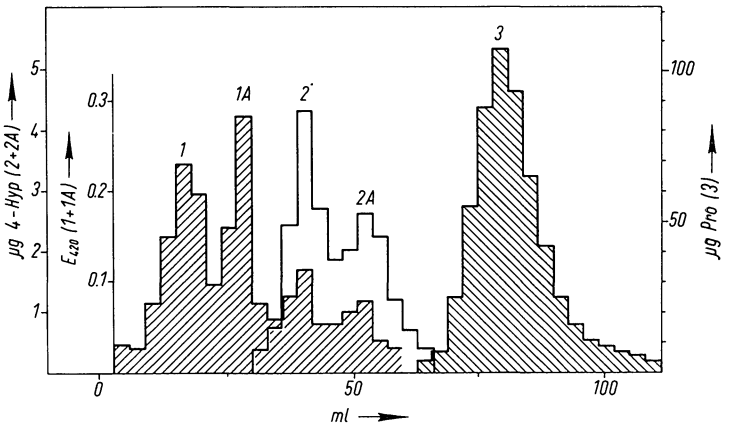

Fig. 2. Isolation of hydroxyproline isomers. Hydroxylation performed in 0.01N HCl, 1mM $FeSO_4$, 10mM ascorbic acid. Onto the column 6 μmoles of proline were introduced.

1: 3-*trans*-hydroxyproline, 1A: 3-*cis*-hydroxyproline (2nd ordinate on the left side);

2: 4-*trans*-hydroxyproline, 2A: 4-*cis*-hydroxyproline (1st ordinate on the left side);

3: proline (ordinate on the right side).

Table 1. Isolation of individual hydroxyproline isomers. For chromatography 6 μmoles of proline were used (s. Fig. 2). The amount of 3-hydroxyproline is only approximate. Applied according to the method of MOORE and STEIN[17], calculated according to calibration curve for 4-hydroxyproline. 4-Hydroxyproline estimated according to STEGEMANN[18].

Experiment No.	Proline reacted [μmoles]	Conversion to hydroxyproline [μmoles]	4-Hydroxyproline total [μmoles]	4-Hydroxyproline *trans* [%]	4-Hydroxyproline *cis* [%]	3-Hydroxyproline total [μmoles]	3-Hydroxyproline *trans* [%]	3-Hydroxyproline *cis* [%]
1	1.54	0.43	0.16	56	44	0.27	57	43
2	1.46		0.13	59	41			

(74% and 27% hydroxyproline yield respectively when compared with 100% yield in the phosphate buffer). In the presence of complex bound iron the hydroxylation occurs in a relatively wide pH range (4.6–7.3) without a marked influence on the amount *(0.28 μmoles) of newly formed hydroxyproline. If* the bivalent iron is free, hydroxylation can be performed too in a less acid milieu and the conversion of proline into hydroxyproline is nearly doubled (Table 2).

Table 2. Formation of hydroxyproline in hydrochloric acid. 20 μmoles of proline were used for incubation, concentration of $FeSO_4$: 1mM; no EDTA was present.

HCl concentr. [N]	0.001	0.005	0.010	0.050	0.100
4-Hydroxyproline [μmoles]	0.27	0.45	0.50	0.16	0.09

Further, the influence of the individual components on the degree of hydroxylation was studied. The course of proline conversion into hydroxyproline dependence on hydrogen peroxide is illustrated in Fig. 3. At higher concentrations of H_2O_2 (40 mM) a secondary reaction:

$$H_2O_2 + {}^{\cdot}OH \rightarrow H_2O + {}^{\cdot}O_2H$$

will take place[19]. The steady state is again the result of the process of synthesis and degradation (curve 1 and 2). It is evident from the figure that hydroxylation of proline occurs also in the absence of metal ions (curve 4). We have no direct evidence about the mechanism of this process. We presume that the active component consists of free radicals which may be the results of the reaction of hydrogen peroxide with ascorbic acid:

$$AH_2 \rightleftharpoons AH^{\ominus} + H^{\oplus}$$

$$AH^{\ominus} + H_2O_2 \rightarrow {}^{\cdot}AH + OH^{\ominus} + {}^{\cdot}OH$$

Fig. 3 shows also the catalytic effect of ascorbic acid (curve 1 and 3) but this component is not an indispensable part of the system and can be substituted by the increasing concentration of $Fe^{2\oplus}$ (Fig. 4, curve 2). As the decomposition of hydroxy-

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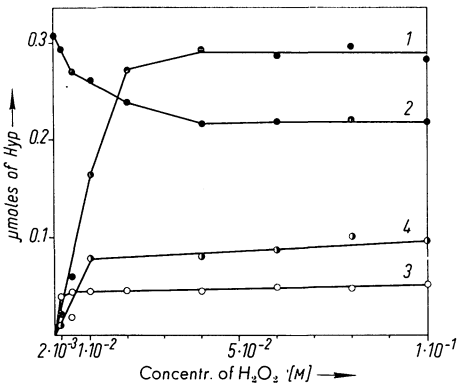

Fig. 3. Effect of hydrogen peroxide concentration on the formation and decomposition of hydroxyproline.

Curve 1: complete hydroxylation system with proline (20 µmoles); Curve 2: complete hydroxylation system with hydroxyproline (0.305 µmoles); Curve 3: proline in the presence of Fe(II)[EDTA] (1mM); Curve 4: proline in the presence of ascorbic acid (10 mM).

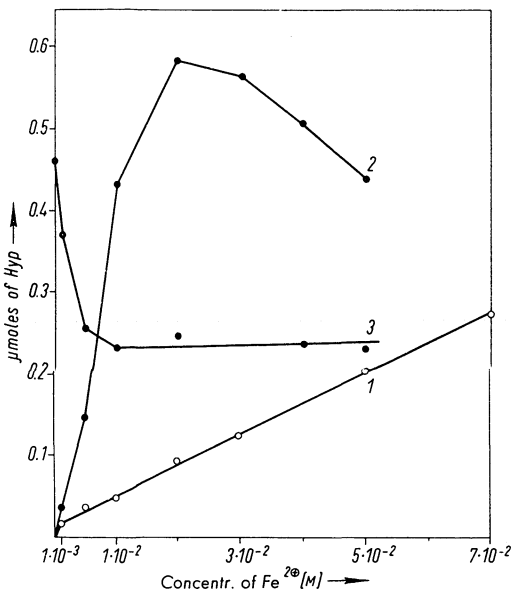

Fig. 4. Effect of concentration and compound of iron ions on formation and decomposition of hydroxyproline.

Curve 1: 20 µmoles proline incubated with $Fe_3(PO_4)_2$; Curve 2: 20 µmoles proline incubated with Fe(II)[EDTA]; Curve 3: 0.462 µmoles hydroxyproline incubated with Fe(II)[EDTA].

proline reaches a steady state (curve 3) the decrease of hydroxyproline formation at high iron concentrations (curve 2) is not caused by the increase of degradation processes but by the decrease of the concentration of the effective component. In these cases the secondary reaction of:

$$Fe(II)[EDTA] + {}^{\cdot}OH \rightarrow Fe(III)[EDTA] + OH^{\ominus}$$

will take place to a greater extent. Hydroxylation proceeds also in a system where iron is bound in a not readily soluble compound $Fe_3(PO_4)_2$. The comparison of Fig. 4 (curve 2) and Table 3 shows that ascorbic acid can be replaced by the stoichiometric amount of bivalent iron only in the range of low concentrations. In the range of higher concentrations the course of both reactions differs. In the case of ascorbic acid excess degradation processes prevail. As in the case of ascorbic acid, the ethylendiaminetetraacetic acid is not an essential part of the hydroxylation system (Table 4). A maximal effect is achieved by the transfer of $Fe^{2\oplus}$ into the complex which will be oxidized more easily due to the lower oxidation potential[20]. The iron ions can be replaced by ions of other metals able to release electrons (Fig. 5). The incubation was performed in the absence of ascorbic acid and with increased concentration of H_2O_2. $Cr^{3\oplus}$ ions are of the same effectiveness as $Fe^{2\oplus}$; $Cu^{\oplus}$ causes roughly a 20% lower conversion of proline. Ions with a higher valency degree ($Fe^{3\oplus}$ and $Cu^{2\oplus}$) have nearly no effect in this system.

Table 3. Effect of ascorbic acid concentration on the formation of hydroxyproline. 20 µmoles of proline, 1mM $Fe^{2\oplus}$.

Ascorbic acid concentr. [mM]	0	1	5	10	15	20	25
4-Hydroxy-proline [µmoles]	0.04	0.10	0.23	0.29	0.31	0.33	0.24

We also studied the hydroxylation of peptides* containing proline because according to the current opinion proline hydroxylation in vivo occurs only after its incorporation into the collagen precursor[21]. In the hydrolysed peptides, proline and hydroxy-

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Table 4. Effect of chelating agent on the hydroxyproline formation. 20 μmoles proline, 1 mM $Fe^{2\oplus}$ and 10 mM ascorbic acid.

EDTA concentr. [mM]	0	0.1	0.3	0.5	1	2	3	4	5
$Fe^{2\oplus}$:EDTA	∞	10:1	10:3	2:1	1	1:2	1:3	1:4	1:5
4-Hydroxyproline [μmoles]	0.12	0.16	0.21	0.26	0.28	0.28	0.28	0.28	0.28

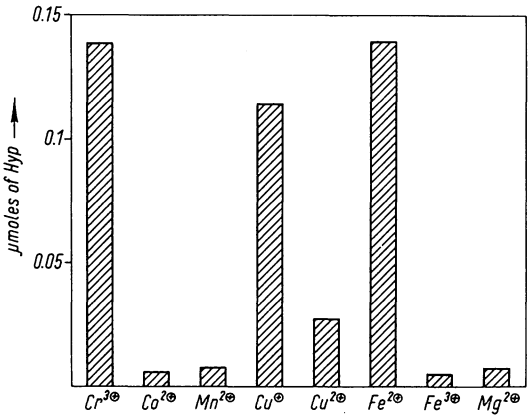

Fig. 5. Comparison of hydroxylation effect of different cations. 20 μmoles proline, 8mM metalions, 100mM hydrogen peroxide, no ascorbic acid. $Cr^{3\oplus}$, $Co^{2\oplus}$, $Mn^{2\oplus}$ in 0.125M NaOH, other metals in phosphate buffer pH 5.6.

Table 5. Hydroxylation of peptides containing proline, incubated under standard conditions.

Substrate	Proline content before incubation [μmoles]	Proline reacted [μmoles]	4-Hydroxyproline formed [μmoles]
Pro	20.42	5.11	0.30
Pro-Gly	20.53	4.51	0.27
Pro-Gly-Gly	19.94	4.59	0.22
Pro-Gly-Gly*	19.40	3.50	0.20
Gly-Pro	19.55	13.67	0.19
Gly-Pro-Leu	20.60	8.24	0.17
Gly-Pro-Leu-Gly-Gly	20.88	8.55	0.15
Gly-Pro-Leu-Gly-Gly*	19.83	9.10	0.14
Gly-Pro-Gly-Pro	18.50	10.70	0.23
Gly-Pro-Leu-Gly-Pro	18.30	8.81	0.23

* differs by provenience.

proline were determined before and after incubation. The results are given in Table 5 and can be summarized as follows:

1. Free proline will be hydroxylated to the maximum extent.
2. Hydroxylation depends on the position of proline. The proline at the carboxyl end of the peptide is hydroxylated the least.
3. The degree of conversion of proline into hydroxyproline decreases with increasing lenghth of the peptide chain.
4. In the presence of 2 proline residues in the peptide, hydroxylation per proline molecule is higher than in peptides with only one proline residue.

We also found hydroxyproline in water solution of proline irradiated by the cobalt bomb (Fig. 6). The amount of formed hydroxyproline is proportional to the radiation dose, i. e. the amount of free radicals formed by water radiolysis. High doses cause again the formation of a steady state. After ninhydrin detection we found by paper chromatography 4 yellow spots corresponding to the 4 hydroxyproline isomers mentioned already before and 2 green spots of unknown products formed probably by polymerization or degradation of the substrate. Furthermore we detected a number of ninhydrin positive substances which confirm the view that the pyrrolidine ring is substantially affected. We did not deal in details with the identification of these reaction products.

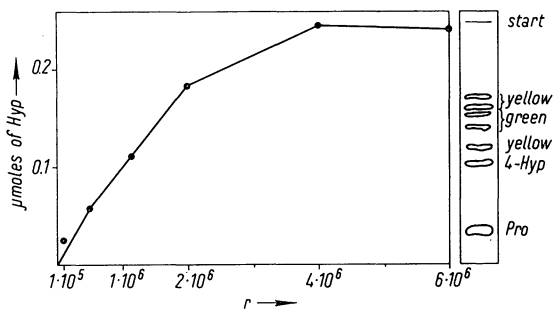

Fig. 6. Effect of the dose of irradiation on hydroxyproline formation. Water solution of proline (10mM) in oxygen atmosphere. For the irradiation by a Co bomb 20 μmoles of proline were used. Chromatogram of irradiated ($6 \cdot 10^6$r) proline solution after detection with ninhydrin.

Discussion

Many papers (for survey cf.[20, 22]) dealing with the mechanism of hydroxylation reactions have been published. UDENFRIEND et al.[2] assume that the product of hydrogen peroxide and dehydro-ascorbic acid is an effective component. But they could not prove the hydroxylation in the presence of synthetically prepared substances of this types. BRAY and GORRIN[23] consider a two electron reduction with formation of $FeO^{2\oplus}$ which is being added to the electronegative position of the aromatic ring:

$$Fe^{2\oplus} + H_2O_2 \rightarrow FeO^{2\oplus} + H_2O$$

$$Fe\ O^{2\oplus} + ArH \rightarrow Fe^{2\oplus} + ArOH$$

ArH = aromatic ring.

Later, GARRICK[24] suggested the following mechanism:

$$Fe^{2\oplus} + H_2O_2 \rightarrow Fe\ OH^{2\oplus} + {}^{\cdot}OH$$

During the decomposition of hydrogen peroxide by iron salts the existence of iron(III)peroxy complexes[25] was proved. Also these substances can be considered as agents transporting the hydroxyl group to the aromatic nucleus.

The majority of authors, however, are of the opinion that it is a question of a non-specific hydroxylation caused by free radicals. The radical mechanism in the simplified system known as FENTON's reagent was presumed by HABER and WEISS[15] and later also by KOLTHOFF et al.[16]. In acid solutions hydrogen peroxide is gradually reduced to water under transitory formation of a hydroxyl radical:

$$Fe^{2\oplus} + H_2O_2 + H^{\oplus} \rightarrow Fe^{3\oplus} + H_2O + {}^{\cdot}OH$$

In the hydroxylation system there are still further reactions such as oxidation of ascorbic acid by hydrogen peroxide and reduction of the metal chelate by ascorbic acid. GRINSTEAD[26] considered the reduction of the metal chelate as the rate determining step. The importance of the chelating agent lies in the fact that it brings the oxidation potential of iron ions into values which are more favourable for a reverse reduction[26]. The mechanism of the effect of this system consists of three parts which proceed simultaneously:

1. formation of effective components (oxidation of $Fe^{2\oplus}$ and ascorbic acid by hydrogen peroxide),
2. regeneration of the system (reduction of $Fe^{3\oplus}$ by ascorbic acid),
3. reaction itself (collision of free radicals with the substrate).

The whole system becomes indistinct and complicated for possible further mutual interactions by free radicals among themselves on the one hand, and free radicals with individual components of the model mixture, on the other hand. The radicals which at the given moment act on the substrate have not been identified as yet. It has to be considered that radicals of ascorbic acid may also take part in the reaction. This is proved by the experimental arrangement with elimination of the metal chelate.

Collision of the free radical with the substrate is followed usually by dehydrogenation[27] which produces a new organic radical:

$${}^{\cdot}OH + RH \rightarrow R^{\cdot} + H_2O$$

This radical can react with hydrogen peroxide:

$$R^{\cdot} + H_2O_2 \rightarrow ROH + {}^{\cdot}OH$$

or may be stabilized in two ways:

$$R^{\cdot} + {}^{\cdot}OH \rightarrow ROH$$

$$R^{\cdot} + {}^{\cdot}R \rightarrow R{-}R$$

Nevertheless in an analogous system:

$$Ti^{3\oplus} + H_2O_2 \rightarrow Ti^{4\oplus} + HO^{\ominus} + {}^{\cdot}OH$$

a transient radical[28] was identified by electron spin resonance spectroscopy formed by addition of ·OH to benzene which further breaks down into phenol and an hydrogen atom:

$$ArH + {}^{\cdot}OH \rightarrow {}^{\cdot}ArHOH \rightarrow ArOH + H$$

For verification of the mechanism of hydroxylation reactions still further proofs must be found.

The results of our experiments such as the time course of hydroxylation, replacement of $Fe^{2\oplus}$ by other metals, appearance of 4 isomers of hydroxyproline and the same yield of 4-hydroxyproline in the model mixture as well as after irradiation of proline water solution by the cobalt bomb presume that there is a non-specific hydroxylation caused by free radicals. Theoretically, 5-hydroxyproline and

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dihydroxyderivatives of proline should also be formed. However, we failed to isolate these substances. In contrast to BRIGGS[13] we cannot confirm the hydroxylation of proline in the presence of $Mg^{2\oplus}$. In agreement with our results LAMPORT[29] isolated as a whole 4 isomers of hydroxyproline whereas CIER et al.[10] found only 4-hydroxyproline.

I wish to express my thanks to Dr. M. CHVAPIL for his interest and valuable discussions, and to Mrs. J. MAŠKOVÁ and Mrs. I. ŠTĚRBOVÁ for their skilled technical assistance.

Experimental

According to the rate, which was found for hydroxyproline formation, the incubation was carried out for 8 min at 37°. The total volume of reaction mixture was 4 ml. The resulting concentrations of the individual components of the hydroxylation system were as follows:

50mM phosphate buffer, pH 5.6,

1mM $FeSO_4$,

4mM disodium salt of ethylenediaminetetraacetic acid,

5mM proline,

10mM ascorbic acid,

50mM hydrogen peroxide.

Peroxide was standardized by the manganometric method. The chemicals used were analytically pure. The water used for preparation of solutions was desaerated by nitrogen. For each experiment fresh solutions were prepared. We used proline from Calbiochem, Los Angeles (NRC Grade, hydroxyproline-free) and its purity was verified chromatographically. Occasionally changed incubation conditions are indicated in the individual tables and figures.

The reaction was stopped by adding 1—2 ml Dowex 50X4 and cooling. After acidification this mixture was introduced to the column of Dowex 50X4 (100—200 mesh, $H^{\oplus}$ cycle, 23.0 × 0.9 cm). After rinsing the column with water the amino acids were eluted by 2N NH_4OH. The appropriate fraction was then concentrated on a water bath, 4-hydroxyproline was determined according to STEGEMANN[18]. For determination of 3-hydroxyproline we used the ninhydrin method according to MOORE and STEIN[17]. The proline was determined according to CHINARD[30] and TROLL and LINDSLAY[31]. The separation of the individual hydroxyproline isomers was made on a Dowex 50WX8 column in $Na^{\oplus}$ cycle (column 70 × 1 cm). For elution a 0.2M citrate buffer, pH 3.5, was used. Fractions of 3 ml were collected.

To check the hydroxylation of peptide bound proline, hydrolysis was performed in the usual manner (105°, 16h, 6N HCl). Before the hydroxyproline isolation the hydrochloric acid was evaporated. Paper chromatography of the irradiated water solution of proline was made on paper Whatman No. 1. A descending technique was used and repeatedly developed in the mixture n-butanol/acetic acid/water 4 : 1 : 5. Ninhydrin was used for detection.

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